REMARKS/ARGUMENTS

Abstract Amendments

The abstract has been amended to avoid the use of legal phraseology as requested by the Examiner. Accordingly, the terms "said" (two occurrences) and "comprising" have been replaced with "the" and "in one aspect, involving", respectively. Also, the Applicants have corrected the abbreviated form for total homocysteine levels by replacing "tHc" with "tHcy" and have corrected the spelling of the full chemical name of Mesna by replacing "2-mercaptoethylsulfonate" with "2-mercaptoethanesulfonate".

The Applicants submit that no new subject matter has been added to the abstract as a result of these amendments. Entry of the abstract amendments is respectfully requested.

Description Amendment

The description has been amended on page 8, line 4 to delete the expression "preventing spread of disease".

The Applicant submits that the description amendment does not add matter to the application. Entry of the description amendment is respectfully requested.

Claim Amendments

Claim 1 has been amended to include the subject matter of claim 6. Claim 6 has therefore been cancelled.

Claim 4 has been amended to replace the comma at its end with a period. This corrects a clerical error.

Claim 7 has been amended to depend on claim 1.

Claims 1-5 and 7-15 are pending in the present application.

The amendments made to the claims have been made without acquiescing to any of the Examiner's objections. Applicant reserves the right to pursue any of the deleted subject matter in a further divisional, continuation or continuation-in-part application.

The Applicants submit that the amendments to the claims do not add new subject matter to the application and that the amended claims submitted herewith are fully supported by the application as filed. Entry of the claim amendments is respectfully requested.

The Official Action dated May 12, 2008, has been carefully considered. It is believed that the amended claims submitted herewith and the following comments represent a complete response to the Examiner's rejections and place the present application in condition for allowance. Reconsideration is respectfully requested.

Election/Restriction

The Examiner has acknowledged Applicants election of Group III, without traverse, in the response to the restriction requirement filed on February 25, 2008. The Applicants elected Group III without traverse because the claims of Groups I and II, namely claims 16-18, had been cancelled in a preliminary amendment dated June 14, 2006.

The Applicants submit that this election without traverse applied only to the election of the invention of Group III, that is to a method of lowering elevated plasma total homocysteine levels in a subject with end stage renal disease. The Applicants did not elect, without traverse, Mesna as the Mesna derivative, as the compound species, nor species (ii-c), Mesna in combination with another type of treatment for a disease associated with elevated plasma thiol levels. The later two selections were merely species elections and at no place in the response dated June 14, 2008, were these elections made without traverse as stated by the Examiner. The Applicants submit that

there is an allowable generic or linking claim and therefore have not withdrawn claims 2 and 15 as suggested by the Examiner.

35 USC § 112, First Paragraph

The Examiner has rejected claim 14 under 35 USC § 112, first paragraph, because the Examiner contends that the specification is not enabling for treatments in the sense of the meaning of preventing the disease.

While not agreeing with the Examiner, to expedite the allowance of this application, the Applicants have amended the specification on page 8, line 4 to delete the expression "preventing spread of disease", thereby rendering the Examiner's rejection moot.

In view of the foregoing the Applicants request that the Examiner's rejection of claim 14 under 35 USC § 112, first paragraph, be withdrawn.

35 UCS § 103(a)

The Examiner has rejected claims 1 and 3-14 under 35 USC § 103(a) as being obvious over Pendyala, et al. Clinical Cancer Research, 2000, 6(4):1314-1321 (herein after "Pendyala") and Cohen, Molecular and Cellular Biochemistry, 2003, 244(1-2):31-36 (herein after "Cohen"), in view of Wilcox, WO 01/30352 A1, 2001 (hereinafter "Wilcox").

The Examiner states that Pendyala teaches that Mesna can reduce cystine and homocystine to cysteine and homocysteine (Hcy), that cysteine and Hcy levels are inversely related to Mesna levels and that these reduced forms are readily cleared by renal excretion. Further, the Examiner states that Cohen teaches that Hcy is a substance known to produce vascular damage and accumulates in subjects with uremia such as those with ESRD and treatments for uremia include dialysis. Finally, the Examiner states that Wilcox teaches that high total plasma homocysteine (t-Hcy) concentration is considered a risk factor for atherosclerosis, occlusive vascular disease and coronary artery disease and because folic acid (a known reducer of t-Hcy

concentration) is used in the treatment of coronary artery disease resulting from hyperhomocysteinemia, and in arterial and venous occlusive diseases and has been studied in athero- and thrombogenesis, there is an implication that reduction of Hcy levels will reduce the risk of cardiovascular related diseases, such as atherosclerosis and venous thrombosis. The Examiner has therefore combined the teachings of Pendyala and Cohen with Wilcox to conclude that it would have been obvious to one of skill in the art at the time of the invention to administer Mesna to a subject, including a human, with end-stage renal disease (ESRD) to lower t-Hcy levels and to combine this Mesna administration with dialysis treatment, conducted during or after Mesna administration.

In the arguments in support of his position, the Examiner states that the motivation to administer Mesna to a subject with ESRD is due to Mesna's art recognized (*c.f.* Pendyala) ability to reduce the amount of Hcy plasma levels and patients with ESRD have elevated plasma Hcy levels and that Hcy is a toxin known to produce vascular damage. The Examiner further states that the motivation to combine Mesna administration with dialysis is because the combination of Mesna with the treatment of dialysis for ESRD would have been complementary treatments to (1) reduce cystine and homocystine to forms more easily cleared by renal excretion in normally functioning kidneys and (2) dialysis would have taken the place of the non-functioning kidneys in the patients with ESRD for removal of the toxic materials in the blood. The Applicants respectfully disagree for the reasons that follow.

Applicants have amended claim 1, and accordingly, claims 2-5 and 7-15 dependent thereon, to specify that the method includes performing dialysis on the subject with ESRD.

As taught in the present application as filed (see for example, page 2, lines 10-17), plasma Hcy is 70-80% covalently bound via a disulfide bond to the cysteine-34 residue of albumin. To lower the total plasma Hcy levels, using the method of the present

application, Mesna is used to exchange with the Hcy on Hcy-cys-34 albumin thereby releasing free Hcy (reduced and mixed disulfide forms), which, in patients with normal renal function, can be eliminated in the urine. The Applicants were able to further show, for the first time, that Mesna is removed from its bound form in the plasma and is eliminated by dialysis. A similar strategy was previously attempted using N-acetylcysteine, however, in patients on chronic hemodialysis it was not successful (see Friedman, et al., Am. J. Kidney Dis. 2003, 41:442-446; copy attached). This contrasts with a study on healthy subjects where N-acetylcysteine was able to lower t-Hcy (see Ventura et al. Pharmacology, 2003, 68:105-114; copy attached). This highlights the surprising and unexpected finding described in the present application that Mesna was able to decrease post-dialysis t-Hcy while, it itself is also removed from the plasma during dialysis, in patients with ESRD.

The Examiner's arguments are based on the assumption that dialysis is a surrogate for the functional kidney. The Applicants submit that it is well known in the art that this is not the case. Patients with ESRD (and essentially no residual kidney function) lose not only glomerular filtration (measured as Glomerular Filtration Rate or GFR) but also many other physiological processes that govern fluid and electrolyte balance. For example, there are several enzymes housed within the kidney that mediate metabolism of drugs and endogenous molecules (including homocysteine). Further, solute transporters in the kidney mediate secretion and reabsorption of solutes. Dialysis does not encompass any of these basic functions in the kidney and therefore not all toxins or other molecules will be removed by this process. As a representative example, phosphorus levels remain elevated in ESRD patients despite dialysis (see, for example, Kuhlmann, M.K. Hemodialysis International, 2006, 10:338-345; in particular page 339, column 1, first paragraph; copy attached).

The interreaction of thiols with albumin involves a complex nucleophilic substitution reaction owing to the uniquely low pKa and inaccessibility of the thiol group of cysteine-34 of albumin (see Sengupta et al. Journal of Biological Chemistry, 2001, 276:30111-

30117). Pendyala merely provides *in vitro* evidence that Mesna can reduce cystine and homocystine to cysteine and homocysteine, respectively (see page 1319, right column, paragraph 3 of Pendyala). The Applicants note that homocystine itself is a dialyzable molecule and its reduction to homocysteine would not be expected to have any effect on dialytic excretion. Therefore, while Pendyala teaches that Mesna improves renal excretion of Hcy in cancer patients also being treated with ifosamide, the use of Mesna in patients with "end stage" kidney function to liberate Hcy from plasma protein and render it removable by dialysis, as well as the subsequent liberation and removal of Mesna by dialysis, is not at all implied by Pendyala, either alone or in combination with Cohen and Wilcox.

In the PTO's own Obviousness Guidelines, the rules for making an obviousness rejection based on the obvious to try reasoning includes the provision that there be a finding that one of ordinary skill in the art could have pursued known potential solutions with a reasonable expectation of success. As noted above, a person of skill in the art would not have a reasonable expectation that Mesna could successfully liberate Hcy and itself be liberated and removed by dialysis in a patient with ESRD. In fact, based on the negative results obtained with N-acetylcysteine, a person skilled in the art would have the expectation that this method would not be successful since the results reported in Friedman and Ventura teach away from using this approach.

Finally, the Applicants wish to point out that hyperhomocysteinemia in ESRD patients has been appreciated since before 1980 and has been a hot topic in the area of research and medicine since the mid-1990's. Pendyala's publication in 2000 disclosed Mesna's ability to reduce thiols. If it were obvious to combine Mesna administration with dialysis for the treatment of ESRD, then the Applicants submit that people skilled in the art would have done this well before the Applicants' first patent submission in December of 2001. This provides further evidence, based on objective indicia, of the non-obviousness of the present invention. Objective indicia are permitted considerations in

any obviousness analysis as outlined in *Graham v. John Deere Co.*, 383 U.S. 1, 17-18 (1966) and as upheld in *KSR Intern. Co. v. Teleflex Inc.*, 127 S.Ct. 1727 (2007).

In view of the above amendments and arguments the Applicant requests that the Examiner's rejection of claims 1 and 3-14 under 35 USC 103(a) be withdrawn.

In view of the foregoing, we respectfully submit that the application is in order for allowance and early indication of that effect is respectfully requested. Should the Examiner deem it beneficial to discuss the application in greater detail, he is kindly requested to contact Patricia Folkins by telephone at 416-957-1686 at his convenience.

The Commissioner is hereby authorized to charge any deficiency in fees or credit any overpayment to our Deposit Account No. 02-2095.

Respectfully submitted,

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Albumin Thiolate Anion Is an Intermediate in the Formation of Albumin-S–S-Homocysteine*

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An elevated concentration of plasma total homocysteine is an independent risk factor for cardiovascular disease. Greater than 80% of circulating homocysteine is covalently bound to plasma protein by disulfide bonds. It is known that albumin combines with cysteine in circulation to form albumin-Cys^{34}-S–S-Cys. Studies are now presented to show that the formation of albumin-bound homocysteine proceeds through the generation of an albumin thiolate anion. Incubation of human plasma with L-^{35}S-homocysteine results in the association of >90% of the protein-bound ^{35}S-homocysteine with albumin as shown by nonreduced SDS-polyacrylamide gel electrophoresis. Treatment of the complex with β-mercaptoethanol results in near quantitative release of the bound L-^{35}S-homocysteine, demonstrating that the binding of homocysteine to albumin is through a disulfide bond. Furthermore, using an *in vitro* model system to study the mechanisms of this disulfide bond formation, we show that homocysteine binds to albumin in two steps. In the first step homocysteine rapidly displaces cysteine from albumin-Cys^{34}-S–S-Cys, forming albumin-Cys^{34} thiolate anion and homocysteine-cysteine mixed disulfide. In the second step, albumin thiolate anion attacks homocysteine-cysteine mixed disulfide to yield primarily albumin-Cys^{34}-S–S-Hcy and to a much lesser extent albumin-Cys^{34}-S–S-Cys. The results clearly suggest that when reduced homocysteine enters circulation, it attacks albumin-Cys^{34}-S–S-Cys to form albumin-Cys^{34} thiolate anion, which in turn, reacts with homocysteine-cysteine mixed disulfide or homocystine to form albumin-bound homocysteine.

Homocysteine is a sulfur-containing amino acid formed during methionine metabolism (1). It is catabolized to cysteine through the transsulfuration pathway, or it may be remethylated back to methionine (2). An elevated level of plasma total homocysteine (tHcy)[1] is a strong independent risk factor for cardiovascular disease (3, 4) and an emerging risk factor for Alzheimer's disease (5, 6). tHcy is the sum of free homocysteine and protein-bound homocysteine. Free homocysteine is made up of reduced homocysteine (–SH) (<1% of tHcy), and low molecular weight oxidized disulfides (–S–S–) forms including homocystine (5–10% of tHcy) and homocysteine-cysteine mixed disulfide (5–10% of tHcy). Greater than 80% of tHcy in circulation is bound to protein by disulfide bonds (7–9). A small amount of homocysteine may also be bound to plasma proteins via amide linkage as a result of homocysteine thiolactone reacting with the ε-amino group of protein lysine residues (10). The upper limit of normal tHcy is ~0.012 mM (11, 12). However, in patients with homocystinuria, tHcy levels approach 0.5 mM (13). The overall *in vitro* binding capacity of human plasma proteins for homocysteine is >0.4 mM (14). Almost all pathophysiology studies utilize free reduced homocysteine (reviewed in Ref. 15), whereas little or no attention has been paid to protein-bound homocysteine, despite the fact that it is the most abundant form of circulating homocysteine both in normal and hyperhomocysteinemic subjects.

Albumin is the most abundant protein in plasma. Typical plasma concentrations range from 0.5 to 0.75 mM, and albumin makes up more than 50% of the total plasma protein (16). It is a nonglycosylated, single-chain polypeptide that is folded into three domains that are structurally defined by 17 intrachain disulfide bonds formed between 34 cysteine residues. Albumin contains one additional cysteine residue at Cys^{34} that does not participate in intrachain disulfide bonding. Albumin Cys^{34} accounts for the bulk of free thiol (–SH) in plasma (17). The crystal structure of human serum albumin shows that Cys^{34} is situated in a partially protected slit in a seven residue turn between helices h2 and h3 in subdomain 1A (18) and sits in a crevice 9.5–10 Å deep (17).

The pK_a of the thiol group of Cys^{34} is abnormally low (~5) (19). This is in contrast to the pK_a (~9) of most of the low molecular weight aminothiols present in plasma. Thus, at physiological pH, albumin-Cys^{34} exists primarily as thiolate anion and is highly reactive with metals, thiols, and disulfides (20). In fact, about one-third of the albumin molecules in the plasma carry disulfide-bonded thiols at this Cys residue (20). These ligands probably become disulfide bonded in the plasma, because the albumin that is formed and secreted from the liver is in the free thiol form (17). Certain drugs containing thiol groups also bind to Cys^{34} of albumin (19, 21, 22). This Cys^{34} of albumin seems

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[1] The abbreviations used are: tHcy, plasma total homocysteine; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-ethanesulfonic acid; DTPA, diethylenetriaminepentaacetic acid; HPLC, high performance liquid chromatography; FD, fluorescence detection.

to be the most probable binding site for low molecular weight thiols including homocysteine. In an earlier study where plasma proteins were resolved by gel filtration chromatography, it appeared that homocysteine was associated with albumin; however, the mechanism of homocysteinylation was not addressed (23). In this study we show that albumin is homocysteinylated when its thiolate anion attacks homocysteine-cysteine mixed disulfide or homocystine.

EXPERIMENTAL PROCEDURES

Reagents—L-Homocystine, L-homocysteine thiolactone, TES, Trizma® base, sodium borohydride, diethylenetriaminepentaacetic acid (DTPA), 5,5′-dithiobis-(2-nitrobenzoic acid), and human serum albumin were purchased from Sigma. Monobromobimane was obtained from Molecular Probes (Eugene, OR). Perchloric acid, HPLC grade acetonitrile, and HPLC grade methanol were from Fisher. All other chemicals used in this study were of reagent grade.

Human Serum Albumin—Crystalline human serum albumin (Sigma; item number A-1653 and lot number 88H7610) was used in these studies. We determined that this albumin preparation contained 0.23 mol -SH/mol protein, 0.33 mol S-S-cysteine/mol protein and 0.015 mol S-S-homocysteine/mol protein. This human serum albumin had 1.5 mol fatty acids/mol albumin. The metal content of this albumin was also determined using inductively coupled plasma mass spectrometry (24). The samples were digested with nitric acid in polytetrafluoethylene test tubes with ^{71}Ga as an internal standard. The albumin was found to contain 3.62 ppm of copper, 191.5 ppm of calcium, 12.96 ppm of iron, 0.015 ppm of cobalt, and 0.85 ppm of nickel. Albumin is also known to carry other thiols (*e.g.* glutathione and cysteinylglycine) along with other metabolites (*e.g.* nitric oxide) on Cys^{34}; however, the concentrations of these compounds were not determined in this study.

Preparation of L-^{35}S-Homocysteine—L-^{35}S-Homocysteine was prepared from L-[^{35}S]methionine as described by Mudd *et al.* (25) with slight modification. Briefly, 0.02 mmol of L-methionine was mixed with 1 nmol of L-[^{35}S]methionine (1 mCi) and refluxed with 5 ml of hydriodic acid for 18–20 h under argon atmosphere to produce L-^{35}S-homocysteine thiolactone. The solution was evaporated to dryness under flowing argon for 24 h. The resulting yellow oily reaction mixture was dissolved in 0.5 ml of water and subjected to descending paper chromatography (Whatman 3 MM) using the solvent system isopropyl alcohol:formic acid:water (70:10:20). The standards of methionine and homocysteine thiolactone were run adjacent to the reaction mixture. Before developing the chromatogram with ninhydrin to locate the amino acid standards, the central portion of the chromatogram was removed. After developing the standard spots, it was repositioned, and the area corresponding to homocysteine thiolactone was marked, cut out, and eluted from the paper with water. The resulting aqueous solution was dried under vacuum and resuspended in water. The concentration of L-^{35}S-homocysteine thiolactone was determined spectrophotometrically at 243 nm using a molar absorptivity of 2.50 M^{-1} cm^{-1}.

L-^{35}S-Homocysteine was prepared from the L-^{35}S-homocysteine thiolactone by the method of Duerre and Miller (26) with slight modification. Briefly, L-^{35}S-homocysteine thiolactone was hydrolyzed with NaOH (5 M) for 5 min at 37 °C to open the thiolactone ring. The solution was then neutralized with 2 N HCl and diluted with TES buffer (0.05 mM, pH 7.4) to obtain the desired stock concentration of L-homocysteine. The concentration of L-homocysteine was determined by the method of Ellman (27). For nonradioactive experiments L-homocysteine was prepared from L-homocysteine thiolactone in the same manner.

Preparation of Homocysteine-Cysteine Mixed Disulfide—Homocysteine-cysteine mixed disulfide was prepared as described by Rudy *et al.* (28). Briefly, homocysteine (20 mM) and cysteine (20 mM) were incubated in the presence of the catalyst, diaquocobinamide, in TES buffer (1 mM, pH 7.4) for 40 min. The reaction mixture was then separated by preparative paper chromatography using the solvent system isopropanol:formic acid:water (70:10:20), and the band corresponding to homocysteine-cysteine mixed disulfide was cut from the paper and eluted with water.

Preparation of Albumin-S⁻ Thiolate Anion—Human albumin thiolate anion (mercaptalbumin) was prepared as described by Sogami *et al.* (29). Briefly, human serum albumin (1 mM) in 0.1 M sodium phosphate buffer (0.3 M NaCl, pH 6.86) was treated with dithiothreitol (final concentration, 5 mM) at 25 °C for 45 min. It was then dialyzed extensively against 0.1 M sodium phosphate buffer (0.3 M NaCl, pH 6.86) at 4 °C to remove dithiothreitol. Under these conditions, none of the 17 intrachain disulfide bonds of human albumin were reduced.

Binding of L-^{35}S-Homocysteine to Human Plasma—Human plasma (0.1 ml) was diluted with 0.1 ml of 0.1 M TES buffer (pH 7.2) in a microcentrifuge tube and preincubated for 10 min at 37 °C before the addition of L-^{35}S-homocysteine (final concentration, 0.5 mM). The reaction mixture was incubated at 37 °C with continuous mixing for 5 h. Plasma proteins were then precipitated using 0.02 ml of 1.5 M perchloric acid. After centrifugation (10 min, 12,000 rpm), the protein pellet was washed three times with 0.1 ml of 1.5 M perchloric acid and the pellet was dissolved in 0.1 ml of nonreducing SDS-polyacrylamide gel electrophoresis sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromphenol blue). To one half of the sample, 0.009 ml of β-mercaptoethanol was added and the sample was boiled for 5 min at 100 °C to reduce disulfide bonds. Aliquots of the β-mercaptoethanol-treated and untreated samples (0.02 ml) were applied to a 10% SDS-polyacrylamide gel and electrophoresed according to the method of Laemmli (30). Gels were dried and analyzed by phosphorimaging to identify the bands corresponding to protein-bound homocysteine.

Binding of Homocysteine to Albumin—Homocysteine (final concentrations, 0.25–1 mM) was added to 0.75 mM human serum albumin in TES buffer (0.05 M, pH 7.2), and the reaction mixture was incubated at 37 °C in a shaking water bath. Aliquots were withdrawn at various time points and added directly to tubes containing 0.1 ml of 1.5 M perchloric acid to precipitate albumin. The tubes were vortexed, incubated for 10 min on ice, and centrifuged for 10 min at 12,000 rpm. The protein pellet was washed three times with 0.1 ml of 1.5 M perchloric acid. The washed pellet was then solubilized in 0.3 ml of Tris buffer (0.5 M, pH 8.5), and the concentrations of albumin-bound thiols were estimated by HPLC, as described below. The perchloric acid soluble fraction was immediately stored at −20 °C. The amount of total free thiol in this fraction was determined using the method of Ellman (27).

Quantification of Homocysteine and Homocysteine-Cysteine Mixed Disulfide—To specifically determine the amount of homocysteine and homocysteine-cysteine mixed disulfide formed during the reaction of human serum albumin with homocysteine, 0.75 mM human serum albumin was incubated with 0.5 mM L-^{35}S-homocysteine. After 3 h of the reaction 50-μl aliquots were withdrawn from the reaction mixture, and albumin was precipitated by adding 1.5 M perchloric acid. The supernatant was subjected to descending paper chromatography using the same conditions as mentioned above. The standards used were homocystine and homocysteine-cysteine mixed disulfide. The areas corresponding to the individual disulfides were cut from the paper and eluted with water, and their radioactivity was determined by counting in a liquid scintillation counter.

Reaction of Albumin Thiolate Anion with Low Molecular Weight Oxidized Thiols—Homocystine (0.125 mM), cysteine (0.125 mM), homocysteine-cysteine mixed disulfide (0.25 mM), or a mixture of homocystine (0.0625 mM) and cystine (0.125 mM) was added to 0.25 mM albumin thiolate anion at 37 °C in a shaking water bath. Aliquots were withdrawn at various time points and added directly to tubes containing 0.1 ml of 1.5 M perchloric acid to precipitate the protein.

HPLC Determination of Thiols—Albumin-bound homocysteine and albumin-bound cysteine were determined by HPLC with fluorescence detection as described by Jacobsen *et al.* (31). Briefly, 0.1 ml of the solubilized albumin pellet (obtained after precipitating the reaction mixture with perchloric acid as mentioned above) was treated with 0.035 ml of 1.43 M sodium borohydride in 0.16 M sodium hydroxide followed immediately by the addition of 0.035 ml of 1.0 M HCl. After addition of 0.05 ml of 7 mM monobromobimane in 50 mM sodium EDTA (pH 7.0), the solution was incubated at 45 °C for 15 min. Albumin was precipitated by the addition of 0.05 ml of 1.5 M perchloric acid. After centrifugation (12,000 rpm, 10 min), the supernatant was adjusted to pH 4 by the addition of 0.025 ml of 2.0 M Trizma® base. The samples (0.10 ml) were then transferred to injector vials for automated HPLC analysis. Standard curves were generated with known amounts of cysteine and homocysteine to calculate the concentrations of the two thiols in the reaction mixture. Albumin concentration was determined by the bicinchoninic acid method (32).

RESULTS

Identification of Albumin as a Binding Protein for Homocysteine in Human Plasma—We recently determined the equilibrium binding capacity of plasma proteins for homocysteine but did not identify the [illegible] binding